REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	02/28/02	Final Progress Report 3/1/01-2/28/02

4. TITLE AND SUBTITLE

In Vivo Incorporation of Unnatural Amino Acids Into Proteins

5. FUNDING NUMBERS

DAAD19-00-1-0014

6. AUTHOR(S)

Dr. Peter G. Schultz

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Scripps Research Institute
10550 North Torrey Pines Road
La Jolla, CA 92037

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U. S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

ARO Proposal # 41085-LS

11. SUPPLEMENTARY NOTES

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

A method for the site-specific incorporation of unnatural amino acids into proteins *in vivo* would significantly facilitate studies of the cellular function of proteins, as well as make possible the biosynthesis of unnatural polymers and proteins with novel structures and activities. Our approach consists of the generation of amber suppressor tRNA/aminoacyl-tRNA synthetase pairs that are not catalytically competent with all the endogenous *Escherichia coli* tRNAs and aminoacyl-tRNA synthetases, followed by directed evolution of such orthogonal aminoacyl-tRNA synthetases to alter their amino acid specificities. A new orthogonal suppressor tRNA/aminoacyl-tRNA synthetase pair in *E. coli* has been derived from the *Saccharomyces cerevisiae* $tRNA^{Asp}$ and aspartyl-tRNA synthetase, and the *in vitro* and *in vivo* characteristics of this pair were determined. In order to achieve a high specificity for the amino acid, a direct selection for site-specific incorporation of unnatural amino acids into a reporter epitope displayed on the surface of M13 phage has been developed and characterized. Under simulated selection conditions, phage particles displaying aspartate were enriched over 300-fold from a pool of phage displaying asparagine using monoclonal antibodies raised against the aspartate-containing epitope. The direct phage selection offers very high specificity for the amino acid of interest, which cannot be achieved by conventional methods.

14. SUBJECT TERMS

15. NUMBER OF PAGES
2

16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

20030515 202

REPORT DOCUMENTATION PAGE (SF298)
(Continuation Sheet)

The method for isolation of a mutant aminoacyl-tRNA synthetase (aaRS) that is specific for an unnatural amino acid from a large pool of aaRS mutants must be: (i) sensitive since the mutants from the initial rounds could have very low levels of activity; (ii) applicable to a wide range of amino acids, i.e., a selection that takes advantage of unique chemical reactivity of certain amino acid side chain would be of limited use; (iii) applicable to large libraries - the desired library size for most *in vivo* selections in *E. coli* is about 10^9 members as determined by the plasmid transformation efficiency or the *in vitro* phage packaging efficiency; (iv) specific, i.e., capable of excluding mutants with broader substrate specificities; and (v) tunable, since the ability to control the stringency of the selection from one round to the next may be important.

We designed and validated a selection that takes advantage of monoclonal antibodies specific for an unnatural amino acid presented in the context of a synthetic immunogenic peptide, the poliovirus C3 epitope. A C3 peptide with a TAG stop codon in the middle was fused to the N-terminus of VCSM13 phage coat protein pIII, such that phage production requires suppression of the amber nonsense codon. *E. coli* cells were then transformed both with a phagemid encoding the synthetase library and the orthogonal tRNA, followed by induction of synthetase expression, and infection with the C3TAG phage. Even a small amount of synthetase activity resulted in suppression of C3TAG and display of the amino acid substrate on the phage surface. Moreover, each phagemid carrying the synthetase gene was preferentially packaged in the same phage that displays the amino acid, since VCSM13 phage DNA does not have an intact M13 intergenic region necessary for efficient packaging. Subsequently, the phage pool representing all of the active synthetase genes in the library was incubated with immobilized monoclonal antibodies directed against the unnatural amino acid, in order to isolate only the phage carrying the synthetase with the desired amino acid specificity.

One way to analyze the efficiency of selection methods is to dilute the desired molecular species in an excess of undesired species and determine the enrichment for each method after one round of selection. To determine the enrichment in a model phage selection, we took advantage of the suppression properties of two orthogonal systems characterized previously: the glutaminyl and the aspartyl pairs. The glutaminyl orthogonal pair is composed of the yeast glutaminyl-tRNA synthetase and the yeast $tRNA^{Gln}_{CUA}$; the aspartyl orthogonal pair consists of the yeast aspartyl-tRNA synthetase containing the E188K mutation and the yeast $tRNA^{Asp}_{CUA}$. In a chloramphenicol acetyl transferase suppression assay (in which a TAG amber codon at a position D125 in CAT gene is suppressed using the orthogonal pair), the chloramphenicol IC_{50} value for the optimized Gln pair is 350 μg/mL, and for the Asp pair, it is 60 μg/mL. The weaker activity of the Asp pair relative to the Gln pair approximates the weak activity of a mutant with novel amino acid specificity in a library containing many more active synthetases specific for the native substrate. Therefore, a substantial dilution of cells containing the Asp pair in an excess of cells containing the Gln pair simulates a library containing a weak hit. The activity of the Asp orthogonal pair was used as selectable phenotype in a model selection in order to determine the enrichment properties of the new selection method. We observed an 800-fold enrichment of the Asp cells over the Gln cells after one round of antibody-mediated selection. Under more stringent conditions, where the Gln orthogonal pair was substituted with a natural supE suppressor tRNA, a 300-fold enrichment was observed.

We conclude that the specificity of monoclonal antibodies combined with the ability to link the product of the aminoacylation reaction with the enzyme-encoding DNA through phage affords a powerful selection for evolving the amino-acid binding sites of aminoacyl-tRNA synthetases. A high enrichment of up to 300-fold per round of selection was observed under model conditions that approximate a real selection, which validated the utility of this novel method. The immunoscreen for the unnatural amino acids displayed on phage complements a set of other powerful methods that are currently used in our laboratory to expand the genetic code.